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Mass-Spectral Identification of an Extracellular Protease from *Bacillus subtilis* KCCM 10257, a Producer of Antibacterial Peptide Subtilein

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Abstract An extracellular protease was identified from *Bacillus subtilis* KCCM 10257 by N-terminal sequencing and mass spectral analysis. The molecular mass of the extracellular protease was estimated to be 28 kDa by SDS-PAGE. Sequencing of the N-terminal of the protease revealed the sequence of A(G,S,R)QXVPYG(A)V(P,L)SQ. The N-terminal sequence exhibited close similarity to the sequence of other proteases from *Bacillus sp.* A mass list of the monoisotopic peaks in the MALDI-TOF spectrum was searched after peptide fragmentation of the protease. Six peptide sequences exhibiting monoisotopic masses of 1,276.61, 1,513.67, 1,652.81, 1,661.83, 1,252.61, and 1,033.46 were observed from the fragmented protease. These monisotopic masses corresponded to the lytic enzyme L27 from *Bacillus subtilis* 168, and the Mowse score was found to be 75. A doubly charged Top product (MS) at a m/z of 517.3 exhibiting a molecular mass of 1034.6 was further analyzed by *de novo* sequencing using a PE Sciex QSTAR Hybrid Quadropole-TOF (MS/MS) mass spectrometer. MS/MS spectra of the Top product (MS) at a m/z of 517.3 obtained from the fragmented peptide mixture of protease with Q-star contained the b-ion series of 114.2, 171.2, 286.2, 357.2, 504.2, 667.4, 830.1, and 887.1 and y-ion series of 147.5, 204.2, 367.2, 530.3, 677.4, 748.4, 863.4, and 920.5. The sequence of analyzed peptide ion was identified as LGDAFYYG from the b- and y-ion series by *de novo* sequencing and corresponded to the results from the MALDI-TOF spectrum. From these results the extracellular protease from *Bacillus subtilis* KCCM 10257 was successfully identified with the lytic enzyme L27 from *Bacillus subtilis* 168.

Key words: Extracellular protease, *Bacillus subtilis* KCCM 10257, mass-spectral identification

Microbial extracellular proteases are very important enzymes, which are now widely used in the food, leather, detergent, and pharmaceutical industries. Extracellular proteases are produced from various microorganisms, including the genus *Bacillus* [13, 22, 28, 29, 31], *Serratia* [11, 14, 18], and *Pseudomonas* [5]. Among these strains, the genus *Bacillus* has played a major role in this field, because it produces a variety of extracellular and intracellular proteases [25], which can be used for various purposes. Furthermore, the strain itself has been applied to express some useful proteins and, in some cases, to modify properties of protein by gene manipulation [32].

Two major types of protease are secreted from *Bacilli*; a subtilisin or alkaline protease (APR) and a metallo-protease or neutral protease (NPR). The alkaline proteases secreted from neutrophilic and alkalophilic *Bacilli* are subjects of special interest, because they represent major commercially produced proteolytic enzymes [1, 7, 16]. These proteases exhibit an optimum activity at pH between 9 to 11, and they are inactivated by serine active-site inhibitors, such as phenylmethylsulfonylfluoride (PMSF) and diisopropylfluorophosphate (DFP). In general, these enzymes have molecular masses ranging from 20,000 to 30,000 Da, are stabilized by Ca^{2+}, and exhibit high isoelectric points [2, 8, 11, 17, 23, 25]. Alkaline proteases are stable from pH 5 to pH 10 at low temperatures, but these enzymes are readily inactivated at high temperature and alkalinity in the absence of Ca^{2+} [1, 2]. Subtilisin is one of the well-characterized alkaline proteases from *Bacillus* sp. and is a serine endopeptidase, having a molecular mass of 27,500 Da. There are several analogues of subtilisin, and their protein sequences from different species of *Bacillus* have been determined [16, 20, 26].

A new antibacterial peptide (subtilein) producer was isolated from a fermented food and identified as *B. subtilis* KCCM 10257 in our previous report [23]. Subtilein exhibited a strong growth inhibitory activity against various *Bacillus* sp., including *B. cereus*. The antibiotic characteristics of subtilein may lead to potential use as a natural preservative in foods to prevent bacterial contamination from foodborne

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Bacillus sp. The protease from this strain may be an important characteristic of the strain, because the antibacterial peptide producer can be used in proteineous foods, such as fermented meat and seafoods, and proteases from this strain can affect the quality of proteineous foods. Therefore, the properties of the enzyme should be well understood and analyzed for further application in industrial fields.

The object of this study was to identify an extracellular protease exhibiting major proteolytic activity from *B. subtilis* KCCM 10257 by N-terminal sequencing and mass spectral analysis. This will offer basic information, making it possible to perform further biochemical characterization of this enzyme such as its substrate specificities, optimum conditions for the enzyme activity, and some factors, that affect the enzyme activity. Furthermore, these results are expected to play a role when *B. subtilis* KCCM 10257 and its antimicrobial peptide, subtilein, are applied to proteineous foods.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Assignments of subtilein producer isolated from salted shrimps were carried out, based on several morphological characteristics and biochemical traits such as cell morphology, Gram staining, growth condition, catalase test, V-P reaction, acid and gas production from glucose, hydrolysis of casein, and growth temperature range according to *Bergey's Manual of Determinative Bacteriology* [6]. The strain was tentatively designated as *B. subtilis* and maintained in the Korea culture center of microorganisms (KCCM) with the number of 10257. The subtilein producer, *B. subtilis* CAU131 (KCCM 10257), was maintained at −70°C in LB (Luria-Bertani, Difco, U.S.A.) broth with 50% glycerol. The strain was grown in LB or BHI (Brain Heart Infusion, Difco, Detroit, MI, U.S.A.) broth at 37°C with shaking at 200 rpm for 18–24 h.

Assay of Protease Activity

The measurement of enzyme activity was carried out in 100 μl of reaction mixture containing 20 μl of enzyme and 78 μl of 20 mM Tris-HCl buffer (pH 9.0), using 2 μl of 4 mM N-Suc-Ala-Leu-Pro-Phe-*p*-nitroanilide as a substrate. The assay was performed by measuring the increase in absorbance at 390 nm per min due to hydrolysis and release of *p*-nitroanilide. After addition of substrate and standing for 2 sec, absorbance was measured at 390 nm with a UV/VIS spectrophotometer (Kontron, Italy). One unit of protease activity was defined as an increase of 0.1 at A_{390} for 1 min in the test reaction.

Purification of Extracellular Protease from *B. Subtilis* KCCM 10257

The enzyme was purified by 70% $(NH_4)_2SO_4$ precipitation, Superdex G-75 gel filtration chromatography on FPLC, Mono-Q anion-exchange chromatography, and hydroxyapatite column chromatography. *B. subtilis* KCCM 10257 cells were grown in 1 l of LB medium at 37°C for 18 to 28 h with shaking at 180 rpm. Bacterial cells were removed from the culture by centrifugation at 4,000 rpm for 10 min, 4°C. The protein in the supernatant was precipitated by 70% ammonium sulfate saturation, and the precipitate was collected by centrifugation at 10,000 rpm for 20 min after standing on ice for 20 min. Superdex G-75 gel filtration chromatography was performed on FPLC (Gilson FPLC system, France) with 20 mM Tris-HCl (pH 9.0) buffer. The crude enzyme obtained from the ammonium sulfate precipitation was loaded onto a Superdex G-75 column. The enzyme was eluted with 20 mM Tris-HCl (pH 9.0) buffer, and the flow rate was 1 ml per 1 min. The contents of protein were monitored at 280 nm. The fractions of all peaks were collected in vials (2 ml per fraction) and assayed for the protease activity. The active fractions obtained from Superdex G-75 gel filtration chromatography were pooled, and the pH of this solution was adjusted to 10.0 with 0.1 N NaOH. The protein solution was loaded onto a Mono-Q anion-exchange column that had been equilibrated with 20 mM Tris-HCl (pH 10.0) buffer. Bound proteins were eluted with a linear radient of 0 to 1.0 M NaCl in 20 mM Tris-HCl (pH 10.0) buffer at 1.0 ml/min. The elution was monitored at 280 nm, and 1.0 ml of each fraction was collected. After dialysis of protease fractions against 10 mM sodium phosphate buffer (pH7.0), the dialysate was loaded to the hydroxyapatite column, and the enzyme was eluted with a linear gradient of sodium phosphate formed from 10 mM to 200 mM.

SDS-Polyacrylamide Gel Electrophoresis

The purity of purified extraprotease was tested in SDS-PAGE as described by Laemmli [12]. Gels contained 10% acrylamide and 0.2% bisacrylamide and were stained with Coomassie blue R-250. The molecular mass of purified protease on the gel was calculated by Rf value of standard marker proteins (Sigma, MO, U.S.A., M3913), including bovin serum albumin (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-phosphate dehydrogenase (36,000 Da), carbonic anhydrase (29,000 Da), trypsinogen (24,000 Da), and trypsin inhibitor (20,000 Da).

Sequencing of N-Terminal Amino Acids

The purified protein was separated by electrophoresis on the SDS-PAGE (12%) and electroblotted onto polyvinylidene difluoride membrane (PVDF) as described by Choli *et al.* [3] with 260 mA for 2 h at 4°C. After confirmation of transferred protein by staining with Coomassie Brilliant Blue R-250, the membrane was destained with 50% methanol. After cutting the protein band on the PVDF membrane, the N-terminal sequence of the purified enzyme was determined by a Procise 491, Protein sequencing system (Milligen, Applied Biosystems).

Identification of Protein by Peptide Mass Mapping

Protein spots of interest in the gel were excised, followed by digestion with trypsin (Promega, Madison, WI, U.S.A.) using a standard in-gel-digestion procedure. Extracted tryptic peptides were desalted using a C18 ZipTip (Millipore, MA, U.S.A.) and then eluted with 50% acetonitrile/0.1% trifluoroacetic acid (TFA). One μl aliquot of the eluant was mixed with an equal volume of matrix solution (saturated-cyano-4-hydroxycinnamic acid in 0.1% TFA-50% acetonitrile in water) and analyzed with a Bruker Biflex III (Brucker Daltonics, Bremen, Germany) matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF) equipped with a nitrogen 337 nm laser. The mass spectra were acquired in the reflectron mode. Internal mass calibration was performed with two trypsin autodigestion fragments (842.5 and 2211.1 Da). This procedure typically results in a mass accuracy of 50 ppm or better. Measured monoisotopic masses of tryptic peptides were used as inputs to search human databases or the NCBInr database using the Mascot search engine with a probability-based scoring algorithm (http://www.matrixscience.com). Up to one missed tryptic cleavage was considered in most cases. A mass accuracy of 50 ppm or lower was used for each search. The molecular mass and isoelectric point of each protein determined through the database search were compared to the corresponding values observed by electrophoresis.

De Novo Sequencing of Peptides by Tandem Mass Spectrometry (MS/MS)

Those proteins unidentified by peptide mass mapping alone, because of low "protein scores" (<63 and $p>0.05$, thus statistically not significant), were identified by *de novo* sequencing of peptides followed by a Blast search. Aliquots of tryptic peptides were cleaned and concentrated using POROS R2 resin (Perceptive Biosystems, Framingham, MA, U.S.A.) in a microcolumn following the method described in the Protana manual (Protana, Odense, Denmark). Tryptic peptides were then analyzed by a hybrid nanospray/ESI-Quadrupole-TOF-MS and MS/MS in a QSTAR mass spectrometer (Applied Biosystems Inc., Foster City, CA, U.S.A.). Peptides in 5% formic acid-methanol (50:50) were sprayed from the gold-coated capillary. Argon gas was used as the collision gas. De novo sequencing of peptides was carried out using the QSTAR software (Analyst QS) and confirmed by a manual interpretation of MS/MS spectra.

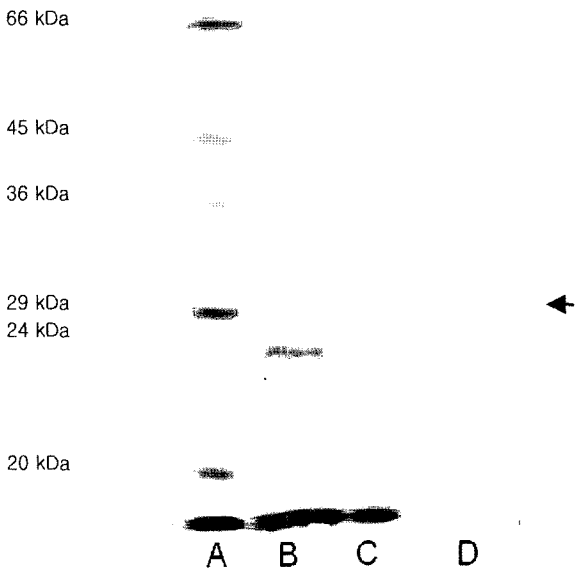

Fig. 1. SDS-PAGE of purified extracellular protease from *B. subtilis* KCCM 10257.
A: Standard marker proteins (bovin serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase; 36 kDa: carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa). B: Fraction after ammonium sulfate precipitation (50 mg). C: Fraction after Mono-Q ion-exchange chromatography (5 mg). D: Purified protease after hydroxyapatite column chromatography (2 mg).

Results and Discussion

Purification of Extracellular Protease from *B. Subtilis* KCCM 10257

Purification of extracellular protease from *B. subtilis* KCCM 10257 was achieved using 70% $(NH_4)_2SO_4$ precipitation, Superdex G-75 gel filtration chromatography on FPLC, Mono-Q anion-exchange chromatography, and hydroxyapatite column chromatography. Active fractions obtained from the Superdex G-75 gel filtration chromatography were rechromatographed on Mono-Q anion-exchange column with a NaCl gradient from 0 M to 1 M. Eluted enzyme fractions exhibited a 16-fold increased specific activity, compared to that of the crude enzyme, and this step was the most effective procedure. The final purification step was hydroxyapatite column chromatography on FPLC and, as shown in Fig. 1, the extracellular enzyme could be purified after this chromatography. The overall purification

Table 1. Purification of extracellular protease from *B. subtilis* KCCM 10257.

Purification step	Total protein (mg)	Toral activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude enzyme	785.36	1,608,768	2,048.45	1.0	100
Superdex G-75	88.87	662,726	7,457.25	3.64	41.19
Mono-Q	10.48	343,014	32,730.34	15.98	21.32
Hydroxyapatite	4.84	228,937	47,301.03	23.09	14.23

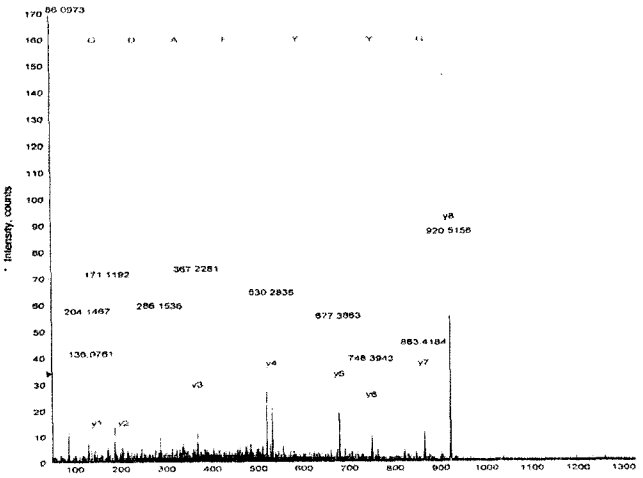

Fig. 2. MS/MS spectra of the doubly charged Top product (MS) at a m/z of 517.3 3 exhibiting a molecular mass of 1,034.6 obtained from a fragmented peptide mixture of protease with the Q-STAR Hybrid Quadropole-TOF (MS/MS) mass spectrometer.

procedure resulted in a 23-fold increase in specific activity with a 14% recovery from the crude enzyme solution. The purified enzyme exhibited a specific activity of 47,300 unit/mg and showed a single band in SDS-PAGE, as shown in Fig. 1. A summary of the purification of protease from *B. subtilis* KCCM 10257 is shown in Table 1. Similar procedures were applied in the purification of other proteases from *Bacillus* sp.: A serine protease was purified from *B. subtilis* KS-1 [27] with 15.4% recovery by using DEAE-Sephadex, Sephadex G-100 chromatography, and an extracellular alkaline protease from *B. subtilis* RM 615 [17] was purified with 23% recovery by using DEAE and CM-cellulose ion chromatographies. Purification of subtilisin J from *B. stearothermophilus* [9] was achieved with 12.8% recovery by using CM-cellulose ion-exchange chromatography.

Comparison of the relative mobility of the enzyme with standard proteins indicated that a molecular mass of the enzyme was approximately 27.5 kDa. In general, the molecular mass of alkaline protease from *Bacillus* ranges from 20 to 30 kDa [4]. The purified protease exhibited the molecular mass similar to other proteases, such as neutral protease (29 kDa) from *B. cereus* [8], alkaline protease (28 kDa) from *B. subtilis* RM 615 [17], keratinolytic serine protease (24 kDa) from *B. subtilis* KS-1 [27], and keratinase from *B. licheniformis* (33 kDa) [15].

Table 3. Summary of the peptide sequences acquired from MALDI-TOF analysis.

Sequence coverage (Matched peptides)	Identified sequences
28%, (6/11)	1276.11: ASVPYGVSQIK 1513.67: APALHSQGFTFSNVK 1652.81: VAVIDSGIDSSHPDLK 1661083: YPSVIAVGAVNSSNQR 1252.61: HPNWTNTQVR 1033.46: LGDAFYYGK

Determination of N-Terminal Sequence

The N-terminal amino acid sequence of purified extracellular protease is summarized in Table 1, and was compared with those of other proteases from *Bacillus* sp. As shown in Table 1, the N-terminal sequence of protease from *B. subtilis* KCCM 10257 was identified as (Ala, Gly, Ser or Arg)-Gln-X-Val-Pro-Tyr-(Gly or Ala)-(Val, Pro or Leu)-Ser-Gln. In the sequencing data, the 1st, 7th, and 8th positions were uncertain. There is some homology in this region among several proteases from *Bacillus* sp., such as proteases from *B. subtilis* KS-1 [27], *B. subtilis* Y-108 [31], and *B. subtilis* Carlsberg [19]. Other proteases such as subtilisin E from *B. subtilis* [26] and subtilisin BPN from *B. amylosacchariticus* [19] exhibited similar results. In most cases, amino acids in the 1st, 2nd, 4th, 5th, and 7th positions exhibited strict homology among the N-terminal sequences of the strains tested. Therefore, from the results of the homology search with the sequences of other proteases from *Bacillus* sp., amino acids at the 1st, 7th, and 8th positions of the N-terminal of protease from *B. subtilis* KCCM 10257 can be suggested as Ala, Gly, and Val, respectively. This result suggests that the extracellur protease from *B. subtilis* KCCM 10257 was very close to the protease from *B. subtilis* Y-108, because the amino acid sequence in the N-terminus matches exactly with the sequence of protease from *B. subtilis* Y-108, except the unidentified amino acid in the 3rd position.

Table 2. N-terminal amino acid sequences of protease from *B. subtilis* KCCM 10257 and other strains.

Bacillus sp.	N-terminal amino acid sequence of protease
Bacillus subtilis KCCM 10257	(Ala)-Gln-X[a]-Val-Pro-Tyr-(Gly)-(Val)-Ser-Gln(Gly) (Ala)-(Pro)(Ser) (Leu)(Arg)
Bacillus subtilis KS-1	Ala-Gln-Pro-Val-Pro-Trp-Gly-Ile-Ser-Gln
Bacillus subtilis Y-108	Ala-Gln-Ser-Val-Pro-Tyr-Gly-Val-Ser-Gln
Bacillus subtilis Calsberg	Ala-Gln-Thr-Val-Pro-Tyr-Gly-Ile-Pro-Leu
Bacillus subtilis var. *amylosacchariticus*	Ala-Gln-Ser-Val-Pro-Trp-Gly-Ile-Ser-Arg
Bacillus subtilis (subtilisin E)	Ala-Gln-Ser-Val-Pro-Trp-Gly-Ile-Ser-Gln
Bacillus amylosacchariticus (subtilisin BPN)	Ala-Gln-Ser-Val-Pro-Trp-Gly-Val-Ser-Gln

[a] X; undetected amino acid.

Table 4. y- and b-ion series from MS/MS spectra of the Top product (MS) at a m/z of 517.3 3 exhibiting a mass of 1,034.6 Da obtained from the fragmented peptide mixture of protease with the Q-STAR Hybrid Quadropole-TOF (MS/MS) mass spectrometer.

	1	2	3	4	5	6	7	8
y ions	147.5	204.2	367.2	530.3	677.4	748.4	863.4	920.5
b ions	114.2	171.2	286.2	357.2	504.3	667.4	830.1	887.1
Amino acids	L	G	D	A	F	Y	Y	G

Mass Spectrometric Analysis of Peptides

The spot of extracellular protease, exhibiting a hydrolytic activity against artificial peptide, was excised from the SDS-PAGE gel. This protein spot was further digested with trypsin as illustrated in Methods and identified using a mass spectrometer. A mass list of the monoisotopic peaks in the MALDI-TOF spectrum was searched after peptide fragmentation. A mass spectrum was acquired from these fragmented peptides, and monoisotopic masses of tryptic peptides measured were used as inputs to search the NCBInr database using the Mascot search engine with a probability-based scoring algorithm. Six peptide sequences exhibiting monoisotopic masses of 1,276.61, 1,513.67, 1,652.81, 1,661.83, 1,252.61, and 1,033.46 were observed from the fragmented protease, and these monisotopic masses corresponded to theoretical mass values of peptide fragments from the lytic enzyme L27 from *B. subtilis* 168 with the Mowse score of 75.

A doubly charged Top product (MS) at a m/z of 517.3 exhibiting a molecular mass of 1,034.6 was further analyzed by *de novo* sequencing using the PE Sciex QSTAR Hybrid Quadropole-TOF(MS/MS) mass spectrometer. As shown in Fig. 2, MS/MS spectra of the Top product (MS) at a m/z of 517.3, obtained from the fragmented peptide mixture of protease with Q-star, contained b-ion series of 114.2, 171.2, 286.2, 357.2, 504.2, 667.4, 830.1, and 887.1 and y-ion series of 147.5, 204.2, 367.2, 530.3, 677.4, 748.4, 863.4, and 920.5. From the b- and y-ion series, the sequence of analyzed peptide ion was identified as LGDAFYYG and, as shown in Table 3, it corresponded to the expected peptide sequence from the MALDI-TOF spectrum.

Since various extracellular proteases produced from the genus *Bacillus* are now widely used for industrial purposes [25, 28, 29, 33], the discovery and characterization of new enzymes are important subjects in this field. One of the important parameters for the characterization of enzyme is the identification of the protein sequence, and the N-terminal sequence is determined using a protein sequencing system in most cases after purification [3]. However, this step does not impart enough information on proteins, especially in the case of protease from Bacillus, because many proteases from the genus *Bacillus* exhibit strict sequence homology in the N-terminal region [18, 26, 27, 30, 31].

In summary, mass spectral techniques were applied to identify an extracellular protease from *B. subtilis* KCCM 10257, and the extracellular protease was successfully identified as the lytic enzyme L27 from *B. subtilis* 168. It is hoped that this will make it possible to perform biological and biochemical characterization of the enzyme and further its applications.

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References

1. Aunstrup, K. 1979. Production isolation and economics of extracellular enzymes. *Appl. Biochem. Bioeng.* **2:** 27–68.
2. Aunstrup, K., H. Outtrup, O. Andersen, and C. Dambmann. 1972. Proteases from alkalophilic *Bacillus* species. *Proceedings of the Fourth International Fermentation Symposium.* Society of Fermentation Technology. Kyoto. Japan, pp. 209–305.
3. Choli, T., U. Kapp, and L. B. Wittmann. 1989. Blotting of proteins onto immobilon membranes. *In situ* characterization and comparison with high-performance liquid chromatography. *J. Chromatogr.* **476:** 59–72.
4. Durham, D. R., D. B. Stewart, and E. J. Stellwag. 1987. Novel alkaline and heat-stable serine proteases from alkalophilic *Bacillus sp.* strain GX6638. *J. Bacteriol.* **169:** 2762–2768.
5. Fukushima, J., S. Yamamoto, K. Morihara, Y. Atsumi, H. Takeuchi, S. Kawamoto, and K. Okuda. 1989. Structural gene and complete amino acid sequence of *Pseudomonas aeruginosa* IFO 3455 elastase. *J. Bacteriol.* **171:** 1698–1704.
6. Holt, J. G, N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams. 1994. *Bergey's Manual of Determinative Bacteriology,* 9th Ed. Williams and Wilkins, Baltimore, U.S.A.
7. Horikoshi, K. and T. Akiba. 1982. *Alkalophilic Microorganisms. A New Microbial World.* Springer-Verlag, New York, U.S.A.
8. Jadwiga, K. S. 1997. Purification and partial characterization of a neutral protease from a virulent strain of *Bacillus cereus. Int. J. Biochem. Cell Biol.* **30:** 579–595.
9. Jang, J. S., D. O. Kang, K. S. Park, and S. M. Byun. 1993. Purification and characterization of recombinant *Bacillus stearothermophilus* subtilisin. *J. Kor. Biochem.* **26:** 595–601.
10. Keay, L., P. W. Moser, and B. S. Wildi. 1970. Proteases of the genus *Bacillus.* II. Alkaline proteases. *Biotech. Bioeng.* **12:** 213–249.

11. Kwon, Y. T., S. Y. Moon, J. O. Kim, Y. H. Kho, and H. M. Rho. 1992. Characterization of extracellular proteases from alkalophilic *Vibrio* sp. strain RH530. *Kor. J. Microbiol.* **30:** 501–506.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
13. Lee, M. H., J. J. Song, Y. H. Choi, S. P. Hong, E. Rha, H. K. Kim, S. G. Lee, H. Poo, S. C. Lee, Y. B. Seu, and M. H. Sung. 2003. High-level expression and secretion of *Bacillus pumilus* lipase B26 in *Bacillus subtilis* Chungkookjang. *J. Microbiol. Biotechnol.* **13:** 892–896.
14. Lee, S. H., J. M. Kim, Y. T. Kwon, Y. H. Kho, and H. M. Rho. 1992. Cloning, sequencing and expression of an extracellular protease gene from *Serratia marcescens* RH1 in *Escherichia coli*. *Kor. J. Microbiol.* **30:** 507–513.
15. Lin. X., C. Lee, E. S. Casale, and J. C. H. Shilh. 1992. Purification and characterization of a keratinase from a feather-degrading *Bacillus licheniformis* strain. *Appl. Envir. Microbiol.* **58:** 3271–3275.
16. Markland, F. S. and E. L. Smith. 1967. Subtilisin BPN'. VII. Isolation of cyanogen bromide peptides and the complete amino acid sequence. *J. Biol. Chem.* **242:** 5198–5211.
17. Moon, S. Y., T. K. Oh, and H. M. Rho. 1994. Purification and characterization of an extracellular alkaline protease from *Bacillus subtilis* RM 615. *Kor. Biochem.* **27:** 323–329.
18. Nakahama, K., K. Yoshimura, R. Marumoto, K. Mosakazu, I. S. Lee, T. Hase, and H. Matsubara. 1986. Cloning and sequencing of serratia protease gene. *Nucleic Acids Res.* **14:** 5843–5855.
19. Narida, K. 1979. pp. 212–221. *In*: *Biochemistry Databook*. Kagakidoujin. Tokyo.
20. Nedkov, P., W. Oberthur, G. Baunitzer, and Z. Hoppe-Seyler's. 1983. Primary structure of subtilisin DY. *Physiol. Chem.* **364:** 1537–1540.
21. Ottesen, M. and I. Sveden. 1970. Proteolytic enzymes. *Methods Enzymol.* **19:** 199–215.
22. Paik, H. D., S. K. Lee, S. Heo, S. Y. Kim, H. H. Lee, and T. J. Kwon. 2004. Purification and characterization of the fibrinolytic enzyme produced by *Bacillus subtilis* KCK-7 from Chungkookjang. *J. Microbiol. Biotechnol.* **14:** 829–835.
23. Park, S. Y., Y. J. Yang, Y. B. Kim, J. H. Hong, and C. Lee. 2002. Characterization of subtilein, a bacteriocin from *Bacillus subtilis* CAU131 (KCCM 10257). *J. Microbiol. Biotechnol.* **12:** 228–234.
24. Priest, F. G. 1977. Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol. Rev.* **41:** 711–753.
25. Sloma, A., A. Ally, D. Allay, and J. Pero. 1998. Gene encoding a minor extracellular protease in *B. subtilis*. *J. Bacteriol.* **170:** 5557–5563.
26. Stahl, M. L. and E. Ferrari. 1984. Replacement of the *Bacillus subtilis* subtilisin structural gene with an *in vitro*-derived deletion mutation. *J. Bacteriol.* **158:** 411–418.
27. Suh, H. J. and H. K. Lee. 2001. Characterization of a keratinolytic serine protease from *Bacillus subtilis* KS-1. *J. Protein Chem.* **20:** 165–169.
28. Takagi, M., T. Imanaka, and S. Aida. 1985. Nucleotide sequence and promoter region for the neutral protease gene from *Bacillus stearothermophilus*. *J. Bacteriol.* **163:** 824–831.
29. Vasantha, N., L. D. Thompson, C. Rhodes, C. Banner, J. Nagle, and D. Filpula. 1984. Genes for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a large open reading frame between the regions coding for signal sequence and mature protein. *J. Bacteriol.* **159:** 811–819.
30. Wells, J. A., E. Ferrari, D. J. Henner, D. A. Estell, and E. Y. Chen. 1983. Cloning, sequencing, and secretion of *Bacillus amyloliquefaciens* subtilisin in *Bacillus subtilis*. *Nucleic Acids Res.* **11:** 7911–7925.
31. Yang, J. K., I. L. Shilh, Y. M. Tzeng, and S. L. Wang. 2000. Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme Microbiol. Tech.* **26:** 406–413.
32. Yang, M. J., S. H. Jung, E. S. Shin, J. Kim, H. D. Yun, S. L. Wong, and H. Kim. 2004. Expression of a *Bacillus subtilis* endoglucanase in protease-deficient *Bacillus subtilis* strains. *J. Microbiol. Biotechnol.* **14:** 430–434.
33. Yang, M. Y., E. Ferrari, and D. J. Hemmer. 1984. Cloning of the neutral protease gene of *Bacillus subtilis* and the use of the cloned gene to create an *in vitro*-derived deletion mutation. *J. Bacteriol.* **160:** 15–21.